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DN 96075561 PubMed ID: 7586219
TI VEGF165 expressed by a replication-deficient recombinant adenovirus vector induces angiogenesis in vivo.
AU Muhlhauser J; Merrill M J; Pili R; Maeda H; Bacic M; Bewig B; Passaniti A; Edwards N A; Crystal R G; Capogrossi M C
CS Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA.
SO CIRCULATION RESEARCH, (1995 Dec) 77 (6) 1077-86.

L10 ANSWER 30 OF 38 MEDLINE on STN DUPLICATE 3
AN 96290680 MEDLINE
DN 96290680 PubMed ID: 8730841
TI Liposome-mediated BDNF cDNA transfer in intact and injured rat brain.
AU Iwamoto Y; Yang K; Clifton G L; Hayes R L
CS Department of Neurosurgery, University of Texas Houston Health Science Center, Houston 77030, USA.
NC PO1 NS31998 (NINDS)
RO1 NS21458 (NINDS)
SO NEUROREPORT, (1996 Jan 31) 7 (2) 609-12.

L10 ANSWER 26 OF 38 SCISEARCH COPYRIGHT 2004 THOMSON ISI on STN
AN 1998:368339 SCISEARCH
GA The Genuine Article (R) Number: ZM121
TI Responses of young and aged rat CNS to partial cholinergic immunolesions and NGF treatment
AU Wortwein G; Yu J; ToliverKinsky T; PerezPolo J R (Reprint)
CS UNIV TEXAS, MED BRANCH, DEPT HUMAN BIOL CHEM & GENET, GALVESTON, TX 77555 (Reprint); UNIV TEXAS, MED BRANCH, DEPT HUMAN BIOL CHEM & GENET, GALVESTON, TX 77555; RIGSHOSP, LAB NEUROPSYCHIAT, DK-2100 COPENHAGEN, DENMARK
CYA USA; DENMARK
SO JOURNAL OF NEUROSCIENCE RESEARCH, (1 MAY 1998) Vol. 52, No. 3, pp. 322-333.

L10 ANSWER 24 OF 38 SCISEARCH COPYRIGHT 2004 THOMSON ISI on STN
AN 1999:135300 SCISEARCH
GA The Genuine Article (R) Number: 164UH
TI Nerve growth factor expressed in the medial septum following in vivo gene delivery using a recombinant adeno-associated viral vector protects cholinergic neurons from fimbria-fornix lesion-induced degeneration
AU Mandel R J (Reprint); Gage F H; Clevenger D G; Spratt S K; Snyder R O; Leff S E
CS LUND UNIV, WALLENBERG NEUROSCI CTR, NEUROBIOL SECT, SOLVEGATAN 17, S-22362 LUND, SWEDEN (Reprint); CELL GENESYS INC, DEPT PRECLIN BIOL, FOSTER CITY, CA 94404; SALK INST BIOL STUDIES, GENET LAB, LA JOLLA, CA 92037
CYA SWEDEN; USA
SO EXPERIMENTAL NEUROLOGY, (JAN 1999) Vol. 155, No. 1, pp. 59-64.

Shin-Lin Chen

Nerve Growth Factor Expressed in the Medial Septum Following *in Vivo* Gene Delivery Using a Recombinant Adeno-Associated Viral Vector Protects Cholinergic Neurons from Fimbria-Fornix Lesion-Induced Degeneration

R. J. Mandel,*¹ F. H. Gage,[†] D. G. Clevenger,* S. K. Spratt,* R. O. Snyder,* and S. E. Leff*

*Department of Preclinical Biology, Cell Genesys Incorporated, 342 Lakeside Drive, Foster City, California 94404; and [†]Laboratory of Genetics, The Salk Institute for Biological Sciences, 10010 North Torrey Pines Road, La Jolla, California 92037

Nerve growth factor (NGF) has been shown to support the survival of axotomized medial septal cholinergic neurons after aspirative lesions of the fimbria-fornix (FF). This survival effect has been achieved utilizing intraventricular and intraparenchymal delivery of the NGF protein. While the use of NGF for the treatment of the cholinergic deficits present in Alzheimer's disease shows promise based on its efficacy in animal models, concerns about side-effects of intraventricular NGF delivery in humans have been raised. In the present study, NGF was delivered directly to the medial septum via a recombinant adeno-associated viral vector (rAAV) encoding the cDNA for human NGF prior to a FF lesion in rats. This rAAV-mediated NGF delivery was shown to significantly attenuate the medial septal cholinergic cell loss observed in animals receiving an equivalent injection of a control rAAV vector. © 1999 Academic Press

Key Words: gene therapy; Alzheimer's disease; growth factors; basal forebrain; acetylcholine.

Intracerebral administration of nerve growth factor (NGF) has been proposed as a treatment for the cholinergic-sensitive cognitive symptoms of Alzheimer's disease (31) (AD), because of its ability to spare damaged CNS cholinergic neurons (13, 38), enhance cholinergic parameters in young and aged rats (5, 11, 14, 28, 37), and reverse cognitive deficits in aged rodents (7, 9, 25–27). Unfortunately, identification of hyperplasia of glial cells in the midbrain and spinal cord of animals treated intracerebroventricularly (ICV) with biologically active doses of NGF have halted development of NGF as a therapy for AD (4, 39). While intraparenchymal delivery of NGF maintains biological efficacy and circumvents the problems of ICV delivery (36), this

protein delivery strategy is not practical for clinical use due to potential damage induced by the permanent introduction of a cannula to the brain parenchyma and the need for more diffusion than can be achieved by local protein injection.

Gene therapy (the ability to deliver and express a therapeutic gene locally or systemically) is being developed to deliver genes expressing proteins in the CNS without the permanent introduction of a cannula into the brain parenchyma (10). Thus, intracerebral delivery and expression of an NGF cDNA may be an attractive protein delivery method for the treatment of AD. The present study demonstrates the bioactivity of NGF following rAAV-mediated gene delivery using the fimbria-fornix (FF) model of cholinergic neuronal degeneration.

An rAAV vector expressing the human NGF cDNA was constructed using standard cloning techniques. The coding region of human β NGF was obtained by PCR amplification of previously described genomic clones (ATCC 57488-57491; DNA Sequence Accession No. V01511 (35)). The pSSV9-MFG-s-hNGF plasmid was constructed by digesting plasmid SSV9-MFG-s-K9F9 with Age I + *Bam*HI to remove canine factor IX sequences and inserting the 0.8-kb Age/*Bam*HI fragment containing human β NGF (33). Recombinant AAV (rAAV) vectors were prepared according to Snyder *et al.* (33), with modifications. Briefly, subconfluent 293 cells were cotransfected with the vector plasmid (described above) and the AAV helper plasmid pACG 2-1 (21) using the calcium phosphate method. Cells were then infected with adenovirus Ad5dl312 (an E1A[−] mutant) at an m.o.i. of 2 and the infection was allowed to proceed for 60–72 h. Cells were harvested and three freeze/thaw cycles were carried out to lyse the cells. The nucleic acid in the lysate was digested with 250 U/ml Benzonase (Nycomed) at 37°C for 10 min and then centrifuged at 1500g to pellet the cellular debris. The cell lysate was then fractionated by ammonium sulfate precipitation and the rAAV virions were isolated on two

¹ Present address: Wallenberg Neuroscience Center, Section of Neurobiology, University of Lund, Sölvegatan 17, S-223 62 Lund, Sweden.

sequential continuous CsCl gradients. The gradient fractions containing rAAV were dialyzed against sterile PBS, heated for 45 min at 56°C, and stored at -80°C. The purity of the rAAV virus with regard to cellular contaminants, infectious adenovirus, and contaminating wt-AAV were evaluated as reported previously (33). The vector used for control injections was rAAV-CMV-LacZ (pdx-31 LacZ), which has been also described previously (30). The titers of the rAAV-MFG-hNGF and the rAAV-CMV-LacZ used in this study were 1.7×10^{12} and 1.0×10^{12} particles per ml, respectively, as estimated by dot blot. The ability of the rAAV-MFG-hNGF vector to infect target cells and direct hNGF production was determined by collecting samples from cultures containing 2.7×10^5 HeLa after transduction with increasing particle numbers of rAAV-MFG-hNGF and measuring the NGF levels produced over a 24-h period with a commercially available ELISA kit (Promega, No. G3550) see Fig. 1.

Fischer 344 male rats weighing approximately 220 g were obtained from Harlan-Sprague-Dawley (Indianapolis, IN), housed with access to *ad libitum* food and water on a 12-h light/dark cycle and were maintained and treated in accordance with published NIH guidelines. All surgical procedures were performed with the rats under isoflurane gas anesthesia using aseptic procedures. Fourteen rats received intraseptal injections of either rAAV-MFG-hNGF ($n = 7$) or rAAV-CMV-LacZ ($n = 7$) at two sites (AP + 0.3, Lat - 0.5, DV - 6.5, AP - 0.3, Lat - 0.5, DV - 7.0, where the DV coordinates were measured from the skull). One microliter of vector was injected over 1 min at each site via a continuous infusion system (19). The needle was left in place for 1 min following the cessation of vector injection, retracted 1 mm, and then withdrawn completely from the brain 4 min later. Three weeks after the vector injections all the rats received unilateral aspirative FF lesions as described in detail previously (38) on the same side as the previous vector injections.

Twenty-one days after the FF lesion, the surviving animals were perfused with 4% paraformaldehyde and processed for choline acetyltransferase (ChAT) immunohistochemistry using a monoclonal α -ChAT antibody (Chemicon, Temecula, CA). Forty-micrometer sections from the dorsal hippocampus were mounted on glass slides and process for acetylcholinesterase (AChE) histochemistry (12). The completeness of the unilateral FF transection was confirmed in each animal by observing a near total absence of AChE staining in the dorsal hippocampus (data not shown). Three or four sections (200 μ m apart, or one of every fifth section) through the medial septum anterior to the anterior commissure were mounted and ChAT positive (ChAT⁺) neurons in both hemisphere's were counted by an observer who was blinded to experimental group membership and the experimental hypothesis in question (see Fig. 2 for

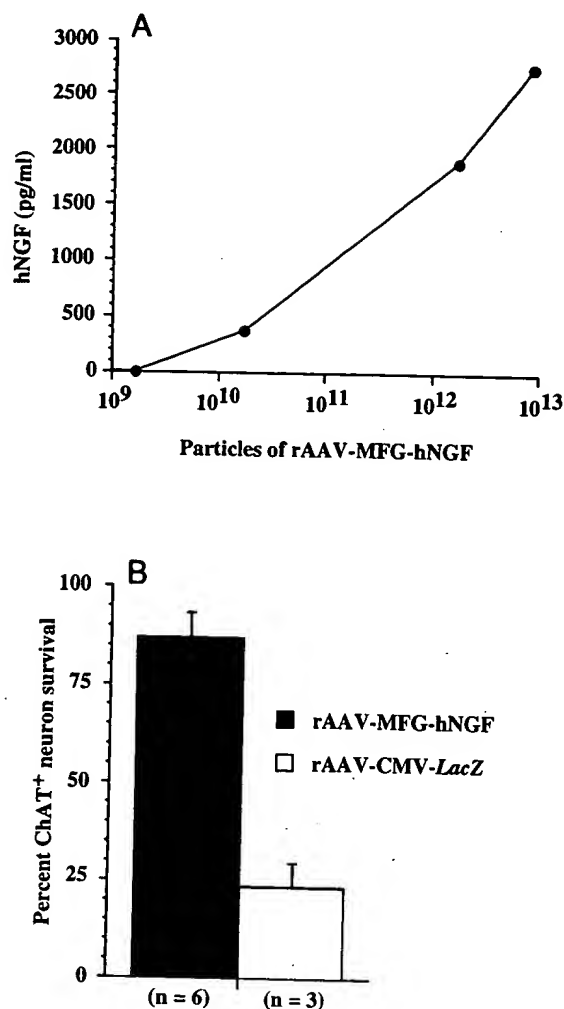


FIG. 1. (A) Production of hNGF by approximately 2.6×10^5 HeLa cells in culture after transduction with increasing particles of added rAAV-MFG-hNGF. The closed circles represent the mean of duplicates of hNGF protein measured in culture media by ELISA. (B) Survival of axotomized ChAT⁺ medial septal neurons after FF lesion. The closed bar indicates the percent survival of the ChAT⁺ medial septal cells of animals that received rAAV-MFG-hNGF (+SEM). The open bar represents the percent survival of the ChAT⁺ medial septal cells of animals that received rAAV-CMV-lacZ control vector injections (+SEM).

sections that are approximately representative of the middle of the counted area). The sections to be analyzed were chosen as described previously (8). Percentage survival of ChAT⁺ medial septal neurons was calculated by dividing the total number of ChAT⁺ cells counted on the lesioned side by the total number of ChAT⁺ cells counted on the intact side ($\times 100$). The probability of the significant difference between the experimental groups was determined by factorial analysis of variance.

In order to confirm that the rAAV-MFG-hNGF vector produced NGF, increasing numbers of particles of the vector were added to cultured HeLa cells. Two days

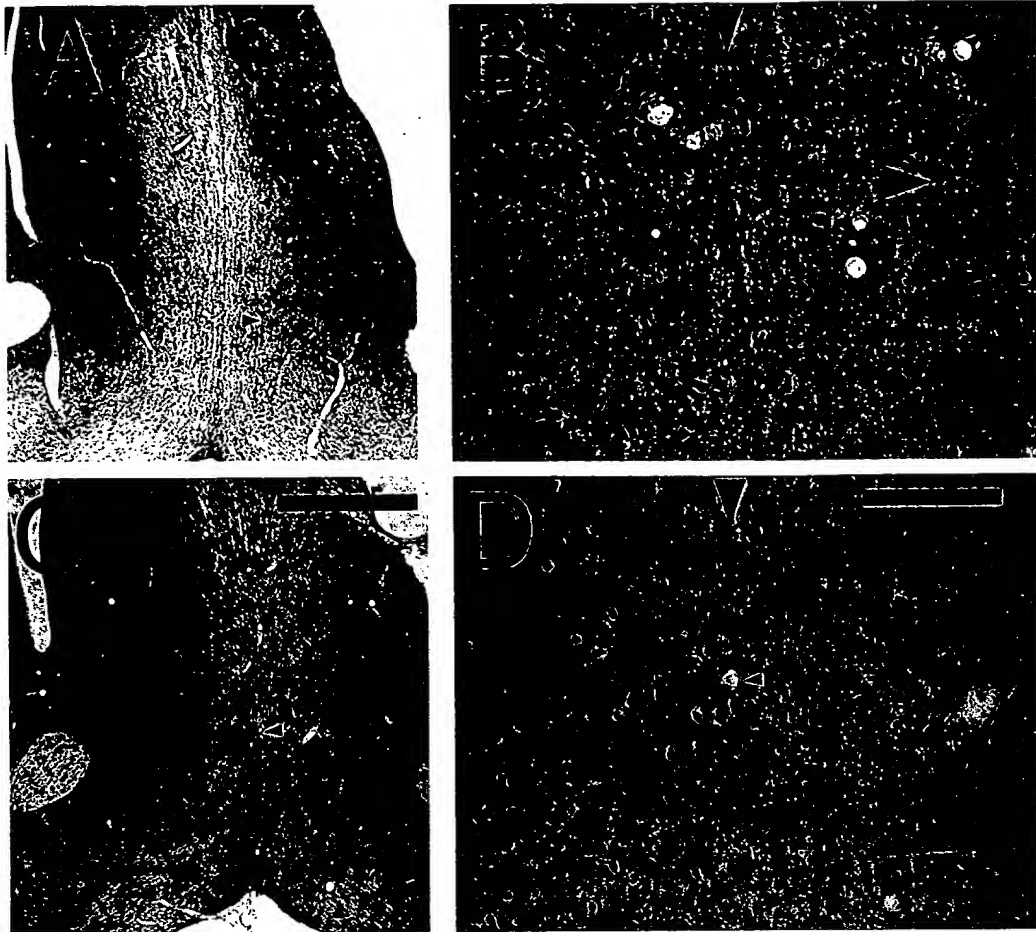


FIG. 2. ChAT immunocytochemistry in medial septum after rAAV vector injection and unilateral FF lesions. The lesioned side is on the right of all four panels. (A) Low power photomicrograph of a ChAT stained section from an animal that received rAAV-MFG-hNGF in the right hemisphere six weeks earlier. The arrowhead pointing toward the right indicates the posterior extent of an injection site. (B) Higher power photomicrograph of the same section shown in A. The ChAT⁺ neurons in both hemispheres appear almost indistinguishable in number and size. The large arrowhead pointing down indicates the position of the midline. (C) Low power photomicrograph of a ChAT stained section from an animal which received rAAV-CMV-LacZ 6 weeks in the right hemisphere previously. The black arrowhead pointing to the left indicates a blood vessel that appears in the upper center of D in order to orient the area of enlargement shown in D. (D) Higher power photomicrograph of the same section shown in C. There are two small vessels in the center of this panel which delineate the midline. The large arrow-head pointing down indicates the position of the midline. ChAT⁺ neurons on the intact (left) side of the midline are large and numerous compared to the few small neurons seen on the lesioned side. The Bar in C, 1 mm and applies to A also; Bar in D, 200 μ m and applies to B also.

after transduction, media containing NGF produced over 24 h was collected and assayed for NGF via ELISA. Increasing multiplicities of infection (m.o.i.) of the rAAV-MFG-hNGF vector resulted in measureable levels of NGF in the media when 1.7×10^{10} particles per 2.5×10^5 cells or greater were added to the cultures demonstrating a vector dose-dependent increase in NGF production (Fig. 1A).

Medial septal injection of rAAV-MFG-hNGF ($n = 6$) resulted in significantly greater ChAT⁺ cell survival after FF lesion (86.5%, Fig. 1B) than similar transductions with rAAV-CMV-LacZ [$n = 3$; 23.3%; $F(1, 7) = 35.6$, $P = 0.0005$, see Fig. 1B]. Figure 2 presents representative photomicrographs of the ChAT immunocytochemistry from both experimental groups which demonstrate

the greater cholinergic cell survival in the group of animals which received medial septal rAAV-MFG-hNGF injections. In addition, ChAT⁺ cells on the lesioned side of rAAV-CMV-lacZ-injected controls appear to be shrunken (Fig. 2D) as compared to the ChAT⁺ cells on the lesioned side of rAAV-MFG-hNGF-treated subjects as reported previously (6). Examination of hematoxylin and eosin-stained brain sections from animals that received either rAAV vector revealed no detectable neuropathology 6 weeks after vector injection.

The present data represent the first report of the use of an rAAV vector encoding hNGF to produce a significant survival effect on axotomized septo-hippocampal cholinergic neurons. Similar rAAV vectors expressing

NGF under the control of the cellular neuron-specific enolase internal promoter have been shown to increase basal forebrain ChAT activity and increased Trk receptor density (18). In that report, neurotrophin transgene expression was shown to last at least 6 months (18). rAAV-mediated transgene expression for up to 1 year has also been reported previously (20, 23) and rAAV vectors expressing human tyrosine hydroxylase under the control of the MFG internal promoter have also been observed to support transgene expression for 1 year (Mandel *et al.*, unpublished observations). Moreover, Chamberlin *et al.* (2) have recently concluded that rAAV-mediated transgene expression is likely to be permanent in the rat CNS. Therefore, continued transgene expression in the present experiment, although not demonstrated, is likely.

While rAAV vectors have been asserted to be relatively nontoxic when injected into the CNS (15, 16, 23, 24), this issue has not been adequately addressed as yet. Although no obvious inflammatory response 6 weeks after injection was detected by examination of nissl-stained sections in the present study, pathological responses to the vector closer to the time of the injection cannot be ruled out. Moreover, the extent of the lesion-induced reduction in medial septal ChAT⁺ cells in the rAAV-CMV-*lacZ* control group, while similar in magnitude to that reported previously (29), is greater than that reported by others (17, 32). The large FF lesion-induced depletion of ChAT⁺ cells (75%) in the rAAV-CMV-*lacZ*-injected controls may be due to the large size of the lesion (see the upper right-hand portion of Fig. 1A), but it cannot be ruled out that there was some toxic effect of the rAAV-CMV-*lacZ* control vector.

In the present study, small (1 μ l) injections of the rAAV vectors were placed near the cholinergic cell bodies in the medial septum and a subsequent unilateral FF transection was performed. Transductions of medial septal cells [most likely neurons (15, 23, 30)] with the rAAV vector encoding the hNGF cDNA produced a highly significant protection of medial septal cholinergic cells as compared to an identical control rAAV transduction (see Fig. 1B). Similar results utilizing lentiviral vectors based on human immunodeficiency virus type I that encode hNGF and BCL-xL have recently been reported (1). Taken together, these reports point out the potential of direct *in vivo* gene delivery to produce sufficient NGF near basal forebrain cholinergic nuclei to provide neurotrophic support similar to that reported for delivery of the NGF protein through a cannula (13, 38).

In addition to *in vivo* gene therapy, *ex vivo* gene delivery using cells engineered, by retroviral vectors to express transgenic NGF, has achieved some success in models of basal forebrain cholinergic pathology (3, 17, 22, 29, 34). *Ex vivo* NGF-transduced autologous fibro-

blasts after FF transection in both rats and primates (17, 22, 34). Moreover, NGF-producing autologous fibroblasts transplanted in the basal forebrain of aged rats have been shown to reduce age-related cognitive deficits (3). Similar data have been collected after transplantation of NGF-transduced CNS-derived neural progenitor cells. For example, basal forebrain transplantation of transgenic NGF producing neural progenitor cells protects against FF-lesion-induced cholinergic degeneration (29), improves age-related cognitive deficits (26, 27), and most recently has been shown to prevent progression of age-related cognitive deficits when placed in middle-aged rats (25). Thus, both *in vivo* and *ex vivo* gene therapy strategies have demonstrated the ability to deliver NGF to the basal forebrain in a biologically relevant fashion and to recapitulate many of the effects previously demonstrated by similar studies of NGF protein injections (5, 7, 9, 11, 14, 37).

Both *in vivo* and *ex vivo* gene delivery have advantages and disadvantages associated with their potential progression from research reagents to treatments for neurological disorders. For example, an obvious advantage of *in vivo* gene delivery strategies is the lack of the cell processing necessary for *ex vivo* gene therapy, which may include expansion, purification, and sterility problems. Moreover, problems with the durability of transgene expression have been reported for cells transduced *ex vivo* by retroviruses (19). On the contrary, *ex vivo* gene delivery strategies have the advantage that the identity of genetically altered CNS cells and the effects of genetic engineering on their normal physiology can be more easily characterized than cells which are transduced *in situ* with direct *in vivo* gene transfer methods.

In conclusion, intracerebral administration of NGF has been identified as a potential treatment for the cognitive deficits in Alzheimer's disease (31). Recent reports have identified significant side-effects associated with chronic ICV administration of NGF in rodents and primates (4, 39). Therefore, if NGF is to be utilized to treat Alzheimer's patients then alternative delivery methods must be identified. The data from the present report and those cited above provide support for gene therapy as such an alternative NGF delivery strategy.

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